

THE EFFECTS OF TEMPERATURE ON GERMINATION AND ETHANOL SOLUBLE CARBOHYDRATE PHYSIOLOGY OF SCLEROTIA OF *CLAVICEPS PURPUREA* (FR.) TUL. FROM THE SOUTH-WESTERN CAPE.

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ABSTRACT

Evidence is presented that although sclerotia of *Claviceps purpurea* from *Pennisetum macrourum* Trin. require a temperature treatment (0-10°C) to activate germination, long durations may reduce final percentage germination. Quantitative studies by means of gas liquid chromatography were made on the alcohol-soluble carbohydrates in sclerotia during dormancy, activation and germination. The carbohydrate physiology is discussed in relation to previous work on dormancy and germination of sclerotia of *C. purpurea*.

UITTREKSEL

DIE INVLOED VAN TEMPERATUUR OP DIE ONTKIEMING EN FISILOGIE VAN OPLOSBAAR KOOLOHIDRAAT VAN SKLEROTIA VAN *CLAVICEPS PURPUREA* (FR.) TUL. VAN DIE SUID-WESTELIKE KAAP.

Bewys word gelewer dat alhoewel sklerotia van *Claviceps purpurea* vanaf *Pennisetum macrourum* Trin. 'n temperatuur behandeling (0-10°C) benodig om ontkieming aan die gang te sit, kan lang periodes die end-persentasie ontkieming verlaag. Deur middel van gas vloeistof chromatografie is kwantitatiewe werk gedoen op die etanol-oplosbare koolhidrate van sklerotia gedurende rus-, aktiverings en ontkiemings periode. Die fisiologie van koolhidrate is dan bespreek in die lig van vorige werk op rustende en ontkiemende sklerotia van *C. purpurea*.

INTRODUCTION

Recent studies (Mitchell & Cooke, 1968a) have indicated that dormancy in sclerotia of *Claviceps purpurea* (Fr.) Tul. is broken by a chilling treatment. Studies were performed on sclerotia from areas where normal frosting was a natural occurrence. Yet, epidemics may occur in regions where frosting is not common. A demonstration of sclerotia germinating without temperature activation has been published (Rajendron, 1966); and Hadley (1968) reported that a cold pre-treatment was not essential even though maximum germination was obtained with continuous incubation at 10°C.

Physiological investigations have shown that lipid utilization and changes in the endogenous carbohydrates take place during germination (Mitchell & Cooke, 1968b; Cooke & Mitchell, 1970). These investigations were carried out using sclerotia of *C. purpurea* collected from a pure stand of *Phalaris arundinaceae* L. Sclerotia were analysed for ethanol soluble carbohydrates by means

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of paper chromatography and total sugars and total polyols were determined quantitatively (Cooke & Mitchell, 1969 and 1970). Further work is needed to substantiate these findings and a quantitative estimation of each component of the ethanol-soluble carbohydrates is necessary.

In this investigation sclerotia of *C. purpurea* from *Pennisetum macrourum* were collected from two regions in the South-western Cape, where frost conditions are not normal, although chilling temperatures do occur during the winter months. Mature sclerotia are seen in the host inflorescence between December and March. They subsequently fall onto the soil surface and are subjected to the normal environmental conditions during autumn and winter (May-October). A study was made of the temperature requirements necessary to break dormancy. Changes in the ethanol-soluble carbohydrates during dormancy, activation and germination using gas liquid chromatography were determined.

MATERIALS

Sclerotia were collected from Betty's Bay (CAPE 3418 BD) and Bain's Kloof (CAPE 3319 CA). Both sites are in a winter rainfall area and have typical fynbos vegetation. At Betty's Bay, the grass was present in marshland approximately $\frac{3}{4}$ kilometres from the coastline, while at Bain's Kloof it was found in small clumps in a valley bottom at an altitude of approximately 500 metres. Sclerotia were collected during February 1970, air-dried at room temperature and stored. The material was used from March to October, 1970.

TEMPERATURE ACTIVATION OF GERMINATION

Sclerotia were counted into batches of twenty, and each was placed on sterile damp filter paper in a closed Petri dish. Batches were incubated at 0°, 5° and 10°C and after 2, 4, 8, 12, 16 and 20 weeks a single batch of Bain's Kloof and Betty's Bay sclerotia was removed from each temperature treatment and sclerotia were allowed to germinate at 20°C. Further batches were given continuous incubation at 15°C and 20°C for 25 weeks. Germination was usually scored at daily intervals and a sclerotium was considered germinated when the first clava had emerged through the outer tissue. The results are expressed in Table 1.

A similar pattern of germination took place as in previous studies (Mitchell & Cooke, 1968a). At 0°, 5° and 10°C high germination levels occurred provided that the treatment was of sufficient duration. No germination occurred after continuous incubation at 20°C. At 15°C first signs of germination took place after 15 weeks, but at the end of the experiment only 10% (Bain's Kloof) and 15% (Betty's Bay) of the sclerotia had germinated.

Effects of Temperature on Sclerotia of *Claviceps Purpurea*

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TABLE 1

Final percentage germination and time (days) at room temperature to reach 50% of final germination after chilling at 0°, 5° and 10°C.

Site	Duration of Chilling	0°		5°		10°	
		Final % germination	Time for 50% of final germination (days)	Final % germination	Time for 50% of final germination (days)	Final % germination	Time for 50% of final germination (days)
BETTY'S BAY	2 wks	0	—	30	24	5	14
	4 wks	10	11	70	13	85	18
	8 wks	55	11	80	15	65	13
	12 wks	75	11	85	8	75	7
	16 wks	60	22	80	15	85	4
	20 wks	20	7	15	3	60	—17
BAIN'S KLOOF	2 wks	0	—	5	42	5	16
	4 wks	0	—	50	17	55	27
	8 wks	50	14	70	9	55	25
	12 wks	75	11	60	23	65	12
	16 wks	80	27	55	17	65	22
	20 wks	45	6	30	40	75	— 6

At 0° and 5°C with increasing duration of incubation from 0 to 12 weeks, final percentage germination increased but at longer durations this resulted in reduced final germination figures. At 10°C after 16 weeks incubation there was 15% (Bain's Kloof) and 25% (Betty's Bay) germination and after 20 weeks incubation 65% (Bain's Kloof) and 55% (Betty's Bay) germinated before the sclerotia were transferred to room temperature.

CHANGES IN ETHANOL SOLUBLE CARBOHYDRATES

Sclerotia from Bain's Kloof and Betty's Bay were incubated on damp filter paper in closed Petri dishes at 5°C for 8 weeks. At the end of this period they were transferred to 20° and allowed to germinate. Further batches from Betty's Bay were incubated in a dormant state at 20°C for 12 weeks, transferred to 5°C for 8 weeks to be activated and then allowed to germinate at 20°C. The germination process was divided into four arbitrary stages (Table 2).

Every 3-4 weeks during dormancy and activation and at every germination stage, samples of sclerotia (180-200 mg fresh mass) were dried at 80°C for 6 hr., reweighed and plunged in 4 cm³ of boiling 80% ethanol. At stage 4 germination, clavae were dissected from the sclerotia and were treated as separate samples. Samples were ground and ethanol soluble sugars were extracted in 120 cm³

TABLE 2
Stages in germination of sclerotia of *C. purpurea*.

STAGE	Time (weeks) after Removal from 5°C	Morphological features
1.	1 }	No external signs of germination First clavae emerging Stromata present
2.	2 }	
3.	3 }	
4.	4 }	

80% ethanol using Soxhlet extractor. Extracts were then reduced at 40°C using a rotary evaporator. The residue was dissolved in water, deproteinized by the addition of barium hydroxide and zinc sulphate (Lewis & Harley, 1965) and deionized by means of a mixture of Dowex 1-8 and Dowex 50 W $\times$ 8 ion exchange resins. The filtrate was reduced to dryness and taken up in 20 cm³ of distilled water.

Sugars were then detected by gas-liquid chromatography. A known volume (approximately 5 cm³) was reduced and as an internal standard 1 mg of erythritol was added to each sample. Preliminary paper chromatography using similar methods as described by Cooke and Mitchell (1969) indicated an absence of erythritol in all the samples. The residue was redissolved in 1.0 cm³ of anhydrous pyridine (kept over KOH pellets).

The soluble carbohydrates were separated as their trimethyl silyl (TMS) derivatives, using a similar method to Sweeley et al (1963). The reaction time was 10 minutes and pyridine was blown off with nitrogen gas. The derivatives were redissolved in 1 cm³ of chloroform and centrifuged. 10 μ l of supernatant was injected by means of a Hamilton microlitre syringe into a Pye series 104 gas chromatograph with a Philips PM8100 pen recorder. The columns contained Chromosorb W80-100 mesh as a solid support with a non-polar liquid phase, S.E. 52 (20% methyl phenyl silicone gum). The samples were run on a programme of 160°C for 15 minutes, followed by an increase of 3°C/minute up to 230°C and then a final isothermal run at 230°C for 36 minutes. Peak areas were determined by means of peak triangulation method. Appropriate markers of known concentration were also injected into the column and in the case of erythritol, arabinitol, fructose (combined anomers), glucose (combined anomers) and mannitol, peak areas were found to be linear in the range 0-1.4 mg/cm³ pyridine. Holligan and Drew (1971) have also demonstrated that calibration curves for a number of sugars are linear, by using the digital integration method. Each component except trehalose was calculated in terms of mole % of either total reducing sugars or total polyols.

Because of the poor detection response of trehalose and as no other oligosaccharide was present, trehalose was determined as the difference between

total sugars and reducing sugars. Total sugars in 1 cm³ aliquots of sample were determined quantitatively by the phenol-sulphuric acid method (Dubois et al, 1956), total reducing sugars using modified Nelson method (Somogyi, 1952) and total polyols using periodate consumption method (Lewis & Smith, 1967). The sugar components of each sample were then expressed as mg/g oven-dry weight.

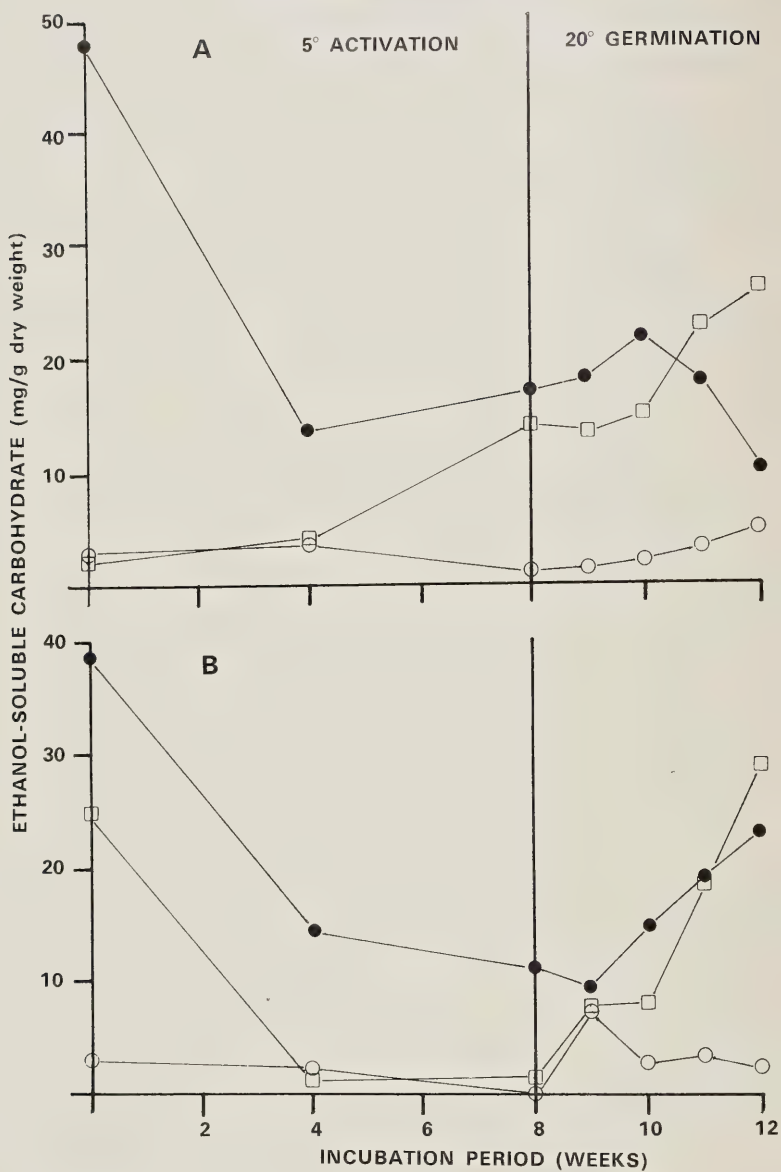
Air-dried sclerotia contained trehalose, mannitol, arabinitol, glucose and fructose and the amounts of each from the two sites are shown in Table 3. Fructose has rarely been found in fungi (Adomako et al, 1971), but further paper chromatography analysis described elsewhere (Cooke & Mitchell, 1969) and the use of p-anisidine hydrochloride as the detection reagent (Hough et al, 1950) indicated the presence of fructose in the air-dried samples.

TABLE 3
Ethanol-soluble carbohydrates of air-dried sclerotia, and samples incubated for 4 weeks at 5°C.

Sites	Sample	Concentration mg/g dry weight				
		Trehalose	Mannitol	Fructose	Glucose	Arabinitol
Betty's Bay	Air-dried	47,90	2,15	8,80	2,73	1,60
	4 weeks at 5°C	13,90	3,99	1,59	3,59	0,16
Bain's Kloof	Air-dried	39,08	24,92	16,01	2,62	18,28
	4 weeks at 5°C	14,52	1,29	1,53	2,19	0,09

Changes during dormancy, activation and germination of trehalose, glucose and mannitol are expressed in Figures 1 and 2, which emphasize previous findings that major changes can be attributed to trehalose and mannitol (Cooke and Mitchell, 1970). Much fructose, arabinitol and trehalose was lost during the initial incubation periods of dormancy and activation. Only one sample contained more than 1,0 mg of fructose/g dry weight after 4 weeks incubation; this was after 8 weeks at 5°C from Bain's Kloof site and 1,59 mg/g was detected. After 4 weeks incubation none of the samples contained more than 0,8 mg arabinitol/g dry weight. There was, therefore, a clear indication of both fructose and arabinitol disappearing very rapidly.

In the Betty's Bay samples there was a steady increase of mannitol during activation, but after removal to 20°C mannitol content of samples from both sites rose. In Betty's Bay samples this increase was from 14,2 to 25,9 mg/g and in the Bain's Kloof samples an increase from 2,0 to 29,1 mg/g was recorded. There were, however, differences in the trehalose contents during germination. In the Betty's Bay samples trehalose declined after 2 weeks germination process



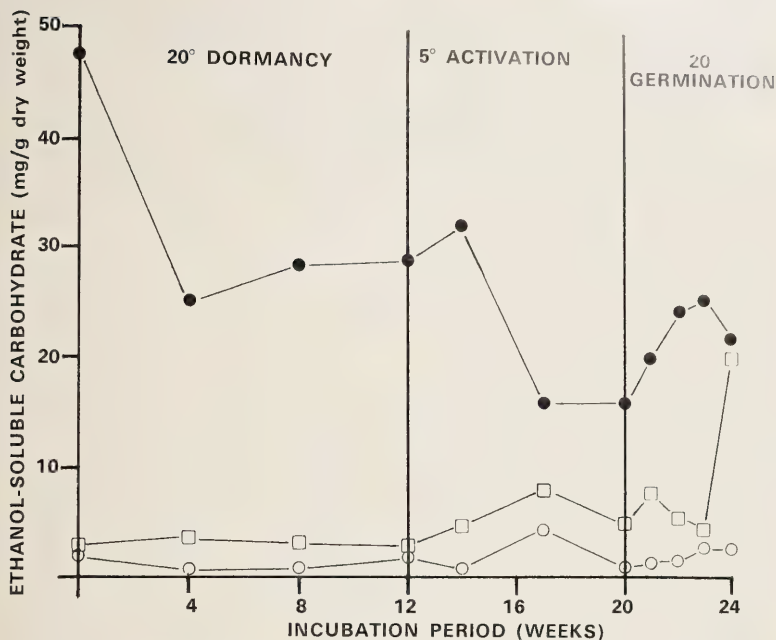


FIG. 2.

Changes in glucose, mannitol and trehalose during dormancy activation and germination in sclerotia of *C. purpurea* from Betty's Bay.

○ — ○ Glucose □ — □ Mannitol
● — ● Trehalose.

from 21.5 to 9.82 mg/g whereas from Bain's Kloof trehalose content increased from 9.51 to 23.0 mg/g after one week's germination process. Dry weight of clavae as a percentage of total (sclerotium and clavae) dry weight and carbohydrate determinations as a percentage of the respective component (sclerotium and clavae combined) are expressed in Table 4. The ethanol-carbohydrate

FIG. 1.

Changes in glucose, mannitol and trehalose in sclerotia of *C. purpurea* during activation and germination.

A — ergots from Betty's Bay
B — ergots from Bain's Kloof
○ — ○ glucose □ — □ mannitol
● — ● trehalose

contents of the clavae were found to be very high which has served to emphasize previous findings (Cooke and Mitchell, 1970), although determinations from S.W. Cape samples were not of the same magnitude.

TABLE 4

Dry weight and soluble carbohydrate contents of clavae in terms of % of total (sclerotium and clavae combined) of respective component.

Site	Dry weight	Trehalose	Mannitol	Glucose
Betty's Bay	18,0	59,6	45,6	42,6
X	11,3	31,8	43,2	39,0
Bain's Kloof	8,1	26,9	23,2	34,3

X—Samples incubated for 12 weeks at 20°C prior to 5°C activation

DISCUSSION

Sclerotia of *C. purpurea* from *P. macrourum* require a chilling treatment to activate germination. An inhibition of germination may occur over longer durations of treatment at the lower ranges of temperature (0°–5°C). At 10°C sclerotia were able to germinate after long durations of treatment before the sclerotia had been transferred to 20°C. Normal weather conditions during the winter months of the south-western Cape suggest that chilling is not frequent and occurs mainly at night time. Thus, in nature, activation of sclerotia may be a cumulative process, but stroma formation of chilled sclerotia takes place only when suitable temperatures (at 10°C and above) prevail for a long enough period.

There are profound changes in the ethanol-soluble carbohydrates during dormancy, activation and germination. The fructose and arabinitol present in sclerotia at the time of their formation disappeared very rapidly with incubation and they may lack an obvious metabolic role (Cooke & Mitchell, 1970). By analogy of lipid metabolism and dormancy of seeds, previous evidence has linked carbohydrate physiology with lipid catabolism (Cooke & Mitchell, 1970). Lipid content of air-dried sclerotia of *C. purpurea* from *P. macrourum* was found to be 48,3% using methods similar to those described in Mitchell & Cooke (1968b), but it is a matter of conjecture whether similar processes are taking place. Supporting the lipid-sugar-mannitol conversion is the rise in mannitol content during germination in sclerotia from both sites, but there were variations in the levels of trehalose. There was an increase in trehalose during germination in samples from Bain's Kloof, compared with a decrease in the samples from Betty's Bay. Glucose content is shown to be fairly steady

during the incubation stages, although any slight changes in glucose appear to be correlated with concomitant changes in trehalose and mannitol. Possibly conversions of glucose to either trehalose or mannitol may be so rapid that it would be difficult to detect, although enzyme studies would determine this. The 'sink' concept proposed by Cooke & Mitchell (1970) appears to be a feasible one in view of the high glucose, trehalose and mannitol contents of the clavae. An energy source is therefore easily available for normal sexual reproduction and formation and ejection of ascospores from the stroma.

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